DNA BARCODING FOR SPECIES IDENTIFICATION IN MALAYSIAN PROCESSED FISH PRODUCTS

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DEDICATION

Specially dedicated to my beloved family, supervisor and friends who have encouraged, guided and inspired me throughout my journey of education.

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For all those who came before, who paved the way, whose footsteps I walk in, and shoulders I stand on.

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ABSTRACT

Currently, high occurrence of mislabelling and species substitution in fish products either intentionally or unintentionally for profit gain has been reported globally, including Malaysia. Such circumstance has urged for increased traceability of fish and the authenticity of raw material to ensure food safety and quality. DNA barcoding offers a rapid, accurate and cost-effective system for species identification via the use of short, standardized mitochondrial marker genes. This study aimed to investigate the prevalence of mislabelling and substitution among fish products in Malaysia market through DNA barcoding approach by targeting the sequence analysis of mitochondrial cytochrome *b* (*Cytb*) gene and cytochrome *c* oxidase subunit I (*COI*) gene. A total of 18 commercial fish products consisting of various processed state were collected from local sources. DNA was successfully extracted from 12 samples (66.67%). Out of the 12 samples, eight samples (66.67%) exhibited successful amplification of *Cytb* barcode (287 bp) by a newly designed primer developed in this study. In contrast, only two *COI* barcodes (~650 bp) from two samples (16.67%) were successfully amplified using fish *COI* universal primer due to its larger amplicon size, suggesting *Cytb* serve as a better DNA barcode marker. In total, 10 barcodes (eight *Cytb* barcodes and two *COI* barcodes) were generated, direct sequenced and compared to BOLD and GenBank database. All products were successfully identified up to species level. The analysis showed that only one (S20) out of eight samples (12.5%) was found to be substituted with a different species (Escolar) which is dangerous for human consumption as it can cause gastrointestinal problems. Furthermore, two eel samples (S17 and S19) were confirmed as threatened species which raise further concern on the trading of processed fish products from the perspective of conservation and highlights the need for the sustainable management of aquatic resources. These findings conclude DNA barcoding as a reliable tool for species identification and suggest *Cytb* could serve as an effective marker for authentication of processed fish products as well as conservation management of fish resources.

ABSTRAK

Kini, kejadian kesalahan label dan penggantian spesies yang tinggi dalam produk ikan sama ada secara sengaja atau tidak sengaja untuk mengautkan keuntungan telah dilaporkan di seluruh dunia, termasuklah Malaysia. Senario sedemikian telah mendorongkan keperluan untuk meningkatkan pengesanan ikan dan ketulenan bahan mentah demi memastikan keselamatan dan kualiti makanan. DNA Barcoding menawarkan satu sistem yang pesat, tepat dan kos efektif dalam pengenalpastian spesies melalui penggunaan gen penanda mitokondria yang pendek dan standard. Kajian ini bertujuan untuk mengkaji kelaziman kesalahan label dan penggantian produk ikan di pasaran Malaysia melalui pendekatan DNA barcoding dengan mensasarkan analisis urutan gen cytochrome *b* (*Cytb*) dan cytochrome *c* oksidase subunit I (*COI*). Sebanyak 18 komersial produk ikan merangkumi pelbagai peringkat pemprosesan telah dikumpulkan dari sumber tempatan. DNA berjaya diekstrak daripada 12 sampel (66.67%). Dari 12 sampel, kod bar *Cytb* (287 bp) berjaya diamplifikasi daripada lapan sampel (66.67%) dengan primer yang direka bentuk dalam kajian ini. Sebaliknya, hanya dua barcode *COI* (~ 650 bp) daripada dua sampel (16.67%) berjaya diamplifikasikan dengan penggunaan *COI* universal primer ikan disebabkan oleh saiz amplicon yang lebih besar, mencadangkan *Cytb* berfungsi sebagai penanda kod bar DNA yang lebih baik. Secara keseluruhannya, 10 kod bar (lapan *Cytb* dan dua *COI*) telah dihasilkan, dijujukan dan dibandingkan dengan pangkalan data BOLD dan GenBank. Semua produk berjaya diidenfikasi ke tahap spesies. Analisis menunjukkan bahawa hanya satu (S20) daripada lapan sampel (12.5%) didapati digantikan dengan spesies lain (Escolar) yang berbahaya untuk penggunaan kerana ia boleh menyebabkan masalah sistem gastrousus. Tambahan pula, dua sampel belut (S17 dan S19) telah disahkan sebagai spesies terancam dan hal ini menimbulkan kebimbangan mengenai perdagangan produk ikan yang diproses dari perspektif pemuliharaan selain menekankan keperluan untuk pengurusan sumber air yang mampan. Penemuan ini menyimpulkan DNA barcoding sebagai alat yang boleh dipercayai untuk pengenalpastian spesies dan mencadangkan bahawa *Cytb* berpontensi untuk diaplikasikan sebagai penanda yang berkesan dalam pengesahan produk ikan yang diproses serta pengurusan pemuliharaan sumber ikan.

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LIST OF ABBREVIATIONS

AFLP	-	Amplified Fragment Length Polymorphism
BLAST	-	Basic Local Alignment Search Tool
BOLD	-	Barcode of Life Database
COI	-	Cytochrome *c* oxidase subunit I
Cytb	-	Cytochrome *b*
DNA	-	Deoxyribonucleic acid
FDA	-	Food and Drug Administration
FSQD	-	Food Safety and Quality Division
FINS	-	Forensically Informative Nucleotide Sequencing
FISH-BOL	-	Fish Barcode of Life Initiative
gDNA	-	genomic DNA
HPLC	-	High Performance Liquid Chromatography
iBOL	-	International Barcode of Life Project
IEF	-	Isoelectric Focusing
INSDC	-	International Nucleotide Sequence Database Collaboration
ITS	-	Internal Transcribed Spacer
IUCN	-	International Union for Conservation of Nature
matK	-	Maturase K
MS	-	Mass Spectrometry
mtDNA	-	Mitochondrial DNA
NJ	-	Neighbour-Joining
NCBI	-	National Center for Biotechnology Information
NMR	-	Nuclear Magnetic Resonance
PCR	-	Polymerase chain reaction
RAPD	-	Random Amplified Polymorphic DNA
rbcL	-	Ribulose-1,5-bisphosphate carboxylate
RLFP	-	Restriction Fragment Length Polymorphisms
SSCP	-	Single-strand Conformation Polymorphism
TAE	-	Tris Acetate EDTA

LIST OF SYMBOLS

bp	-	Base pair
°C	-	Degree Celsius
Kb	-	Kilo base
µL	-	Microlitre
µM	-	Micromolar
mM	-	Millimolar
M	-	Molar
ng	-	Nanogram
ng/uL	-	Nanogram/microliter
nm	-	Nanometre
%	-	Percent
$A_{260/280}$	-	Ratio of the absorbance at 260 nm and 280 nm
rpm	-	Rotary per minute
X	-	Times
U/µL	-	Unit per microlitre
V	-	Volt
w/v	-	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Currently, food authenticity has been a subject of significant concern to food safety authorities due to growing public awareness regarding both the food security and quality (Danezis *et al.*, 2016). Its importance has been highlighted in recent years by a few high-profile incidences of food fraud involving mislabelling species and substitution in the global supply chain, for instance, detection of horsemeat in frozen beef burgers (Boyaci *et al.*, 2014) and detection of donkey meat in salami (Chin *et al.*, 2016). Nonetheless, besides the anxiety of adulteration in meat product, the increasing mislabelling and substitution of fishery products has also pressing great concern over this issue.

Over several decades, the demand of fish has significantly increased due to its nutritional value as part of healthy diet and soon contributed to the expanding trading activity of fish products for worldwide consumption (Fernandes *et al.*, 2017). However, the globalization of fish trade market along with technological advances in food processing, food handling and food transportation by a global network of operators has made the necessity of ensuring the food authenticity and the source of fishery products essential (Maralit *et al.*, 2013). With the incessant occurrence of mislabelling and species substitution in fish (Filonzi *et al.*, 2010; Galal-Khallaf *et al.*, 2014; Carvalho *et al.*, 2015; Cawthorn *et al.*, 2015; Chin *et al.*, 2016; Chang *et al.*, 2016; Christiansen *et al.*, 2018), especially in imported packaged frozen fishery products or highly processed fish products which are indistinguishable based on morphological features, precautionary measures are thus an indispensably necessary (Filonzi *et al.*, 2010). Moreover, ensuring fish authenticity is a great concern not only to avoid commercial fraud, but also for food security to prevent consumption of fish containing species-specific antigens/allergens or toxic compounds which are

detrimental to human health such as pufferfish (*Tetraodontidae*) that causes tetrodotoxin poisoning (Di Pinto *et al.*, 2016) as well as prevention of illegal exploitation of protected species (Chang *et al.*, 2016).

In recent years, molecular biology approaches based on sequencing, specifically the DNA barcoding method, has received considerable attention as a promising tools in fish species identification with its expert-authenticated verification system and high accuracy (Clark, 2015). Mitochondrial DNA genes have arisen as near-universal markers for this purpose (Armani *et al.*, 2017). The mtDNA fragment of cytochrome *c* oxidase subunit I (*COI*) gene or cytochrome *b* (*Cytb*) gene have been widely used as "DNA barcode" for kingdom Animalia discrimination with their high interspecific variation and low intraspecies variation which allow reliable differentiation between species (Hellberg *et al.*, 2017; Filonzi *et al.*, 2010). The launch of Fish Barcode of Life Initiative (FISH-BOL) campaign (www.fishbol.org) as a global effort to coordinate the collection of a standardised reference DNA sequence library for all known fish species and its continuous update further made the identification of fish through this methods available in a much larger scale (Nedunoori *et al*., 2017).

Therefore, with the advancement of DNA barcoding approach together with the continuous expanding of barcode database, Barcode of Life Database (BOLD) and GenBank, the present study attempts of utilizing DNA barcoding as a molecular tool to validate the authenticity of fish products and access their level of misdescription based on mitochondrial *COI* and *Cytb* gene marker, a highly standardization and universality marker for all animals in nature and thus enabling specimens to be identified accurately up to species level.

1.2 Problem Statement

While fish authentication is crucial, the authenticity test and the identification of species is often challenging. Conventionally, the fish species identification is based on morphological characteristics including size, pattern of scale, body shape, number

of fins, measurements of body part and their relative position (Nedunoori *et al*., 2017). Nonetheless, this traditional approach possesses a great limitation when distinguishing and analysing samples which has either significant intraspecific variation or small variability between species even if intact fish exemplar is used (Nedunoori *et al*., 2017). Besides, inspection based on morphological features solely is not suitable when it comes to examining processed fish product such as fish fillet which has lost crucial diagnostic features (Handy *et al.*, 2011).

To counteract the limitation of conventional method, new methods based on molecular genetics has emerged as a more reliable method to apply widely in species identification (Filonzi *et al.*, 2010). Although protein isoelectric focusing (IEF) has been recommended by U.S Food and Drug Administration (FDA) for fish species identification, this technique has limitation in term of not effective for degradative and highly processed specimens (Handy *et al.*, 2011). Hence, DNA barcoding, a species-specific sequence based molecular technique applicable to all kind of products, at the same time, exhibits remarkable accuracy has arisen to be a reliable alternative to address the limitations previous methods encountered.

1.3 Objectives of the study

The objectives of the research are:

(a) To extract genomic DNA (gDNA) from processed fish products.

(b) To amplify the mitochondrial DNA (*Cytb* and *COI* gene) from various processed fish products.

(c) To verify the molecular results of products in species level via bioinformatics analysis.

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